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GlycA, a marker of acute phase glycoproteins, and the risk of incident type 2 diabetes mellitus: PREVEND study

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ABSTRACT

Background: GlycA is a recently developed glycoprotein biomarker of systemic inflammation that may be predictive of incident type 2 diabetes mellitus (T2DM).

Methods: Analytical performance of the GlycA test, measured on the Vantera® Clinical Analyzer, was evaluated. To test its prospective association with T2DM, GlycA was measured in 4524 individuals from the PREVEND study and a survival analysis was performed with a mean follow-up period of 7.3 y.

Results: Imprecision for the GlycA test ranged from 1.3–2.3% and linearity was established between 150 and 1588 µmol/l. During the follow-up period, 220 new T2DM cases were ascertained. In analyses adjusted for relevant covariates, GlycA was associated with incident T2DM; hazard ratio (HR) for the highest vs. lowest quartile 1.77 [95% Confidence Interval (CI): 1.10–2.86, $P = 0.01$], whereas the association of high sensitivity C-reactive protein (hsCRP) with T2DM was not significant. GlycA remained associated with incident T2DM after additional adjustment for hsCRP; HR 1.71 [1.00–2.92, $P = 0.04$]. A multivariable adjusted analysis of dichotomized subgroups showed that the hazard for incident T2DM was highest in the subgroup with high GlycA and low hsCRP ($P = 0.03$).

Conclusions: The performance characteristics of the GlycA test reveal that it is suitable for clinical applications, including assessment of the risk of future T2DM.

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1. Introduction

GlycA is a recently developed nuclear magnetic resonance (NMR) spectroscopy-measured biomarker of systemic inflammation [1–7]. The GlycA NMR signal arises from the N-acetyl glucosamine (GlcNAc) residues on the carbohydrate side-chains of acute phase proteins, mainly α 1-acid glycoprotein (orosomucoid), α 1-antitrypsin, α 1-antichymotrypsin, haptoglobin and transferrin [1,6]. Hence, GlycA is a composite biomarker that integrates both the protein levels and glycosylation states of the most abundant acute phase proteins present in the circulation [1]. GlycA has been demonstrated to be associated with common markers of inflammation such as high sensitivity C-reactive protein (hsCRP), fibrinogen, interleukin-6 and serum amyloid A [1–7]. Recently it was reported that GlycA is related to the leptin/adiponectin

ratio, suggesting that adipose tissue-associated low-grade inflammation could be involved in the regulation of acute phase proteins [5]. Similar to hsCRP, GlycA was found to be higher in subjects with metabolic syndrome and was positively correlated with body mass index (BMI) and insulin resistance as assessed by homeostasis model assessment (HOMA-IR) [4,5,7].

GlycA is elevated in several chronic inflammatory diseases and associated with disease incidence [8–11]. GlycA was elevated in patients with various autoimmune diseases and was associated with disease activity and coronary atherosclerosis in rheumatoid arthritis patients [3,6,12,13]. In the Women's Health Study (WHS) [8], the Prevention of Renal and Vascular End-stage Disease (PREVEND) study [9] and the JUPITER trial [10], GlycA was associated with incident cardiovascular disease (CVD) events, independent of traditional risk factors. GlycA was also found to predict incident type 2 diabetes mellitus (T2DM) in a large population of healthy women [11]. These data raise the possibility that GlycA provides added value for the evaluation of CVD and diabetes risk.

Recently a clinical NMR instrument, the Vantera® Clinical Analyzer, has been developed that addresses the limiting factors of research instruments and allows lipoprotein measurements to be performed in

Abbreviations: GlcNAc, N-acetyl glucosamine; JUPITER, Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin; PREVEND, Prevention of Renal and Vascular End-stage Disease study; RA, rheumatoid arthritis; WHS, Women's Health Study.

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the clinical laboratory [14]. The Vantera Clinical Analyzer, a Food and Drug Administration (FDA)-cleared in vitro diagnostic device, is able to quantify additional analytes from the *NMR LipoProfile*® test spectra, including GlycA and metabolites such as branched chain amino acids [14,15]. Although the GlycA assay has been previously described [1], the analytical performance of the GlycA assay on the Vantera Clinical Analyzer, the instrument that will be reporting GlycA results as a clinical diagnostic test, has not been reported to date.

2. Materials and methods

2.1. Study design

The PREVEND study was approved by the local medical ethics committee, University Medical Center Groningen, The Netherlands; each participant provided written informed consent. Details of the study design and recruitment have been described elsewhere [www.PREVEND.org] [16].

2.1.1. Study participants

Briefly, the PREVEND study is a Dutch cohort drawn from the general population of the city of Groningen. After exclusion of subjects with insulin-treated diabetes and pregnant women, all subjects with a urinary albumin concentration ≥ 10 mg/l were invited to participate ($n = 7768$), of whom 6000 accepted. In addition, a random sample of 2592 individuals with a urinary albumin concentration < 10 mg/l was

included. These 8592 subjects (aged 28–75 years) completed the baseline survey (1997–1998). The second screening, which was the starting point of the current study, took place between 2001 and 2003 ($n = 6894$). GlycA and hsCRP were measured in 5526 subjects from the second screening in whom samples of sufficient quality and quantity were available. For the current study subjects with missing data on diabetes at baseline ($n = 40$), subjects with prevalent T2DM at baseline ($n = 358$) and those with missing data on follow-up ($n = 604$) were excluded, leaving 4524 subjects for the present analysis (Fig. 1).

2.1.2. Follow-up and outcome

Follow-up time was defined as the period between the second screening round (baseline) and the date of ascertainment of T2DM. Follow-up time was censored at 8.5 years. In case a person moved to an unknown destination, census date was date of removal from the municipal registry. Incident cases of diabetes was ascertained if one or more of the following criteria were met: 1) fasting plasma glucose ≥ 7.0 mmol/l (126 mg/dL); 2) random sample plasma glucose ≥ 11.1 mmol/l (200 mg/dL); 3) self-report of a physician diagnosis of T2DM and 4) initiation of glucose-lowering medication use, retrieved from a central pharmacy registry [17,18].

2.1.3. Laboratory analysis in PREVEND

Venous blood was obtained at each screening round after an overnight fast. Plasma samples were prepared by centrifugation at 4 °C. EDTA plasma samples were stored at -80 °C until thawed for

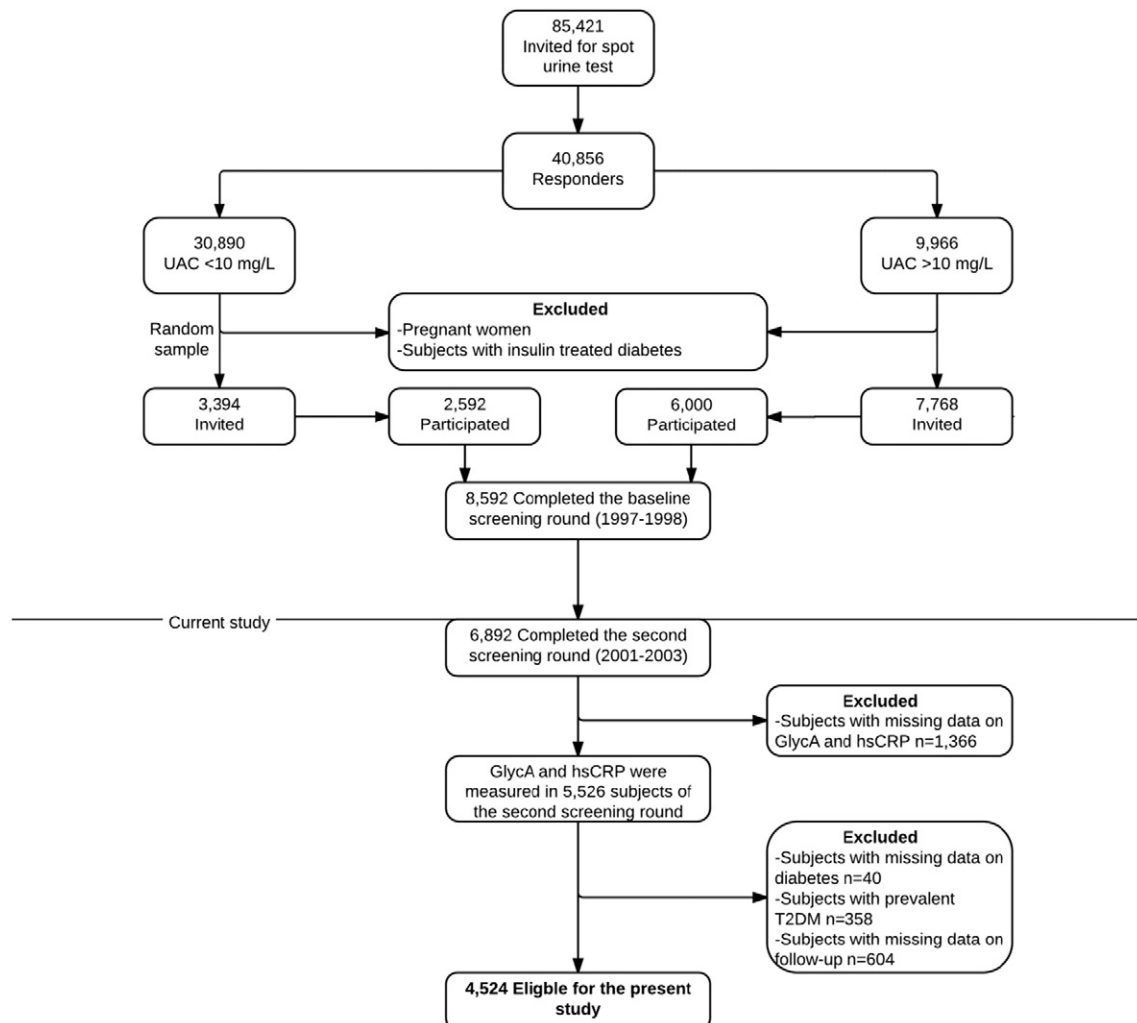


Fig. 1. Flowchart depicting PREVEND participants included or excluded for the purposes of this study.

testing. Plasma samples from the second screening were sent frozen to LipoScience, Laboratory Corporation of America Holdings for testing on the Vantera Clinical Analyzer. NMR spectra were collected and GlycA values were quantified as described above [1]. Total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were measured on a Beckman Coulter® AU680 Analyzer. hsCRP and glucose were determined using standard laboratory protocols [19].

2.1.4. Statistical analyses for the PREVENT study

All statistical analyses were carried out using SPSS ver 22.0. Data are presented as mean \pm standard deviation (SD), median (interquartile range) and percentages. For all analyses, 2-sided $P < 0.05$ was considered statistically significant, except for interaction terms for which the level of significance was set at $P < 0.10$. Baseline characteristics were calculated across sex-stratified quartiles of GlycA. P -values across quartiles of GlycA were determined by linear regression for continuous variables or chi-square test for categorical variables.

Cox proportional hazards regression analysis was performed to examine the associations across quartiles calculated in the whole study population of GlycA and hsCRP with the risk of developing T2DM. In addition, hazards were calculated per 1 SD increment of GlycA and hsCRP. Hazard ratios (HR) were expressed with 95% confidence intervals (CI). TG and hsCRP were log transformed when used as a continuous variable in the analysis. In order to test if each of the biomarkers in the highest range had a different association with incident T2DM vs. both biomarkers or one of these biomarkers in the lowest range the joint associations of GlycA and hsCRP with outcome were evaluated by dichotomizing the distribution of GlycA and hsCRP according to cut points at the highest quartile of GlycA ($>384 \mu\text{mol/l}$) and the highest quartile of hsCRP ($>2.83 \text{ mg/l}$).

Given the enrichment of subjects with microalbuminuria in the PREVENT population, we also performed a secondary analysis in which we accounted for the sampling design of the study, with respect to enrichment of subjects with a urinary albumin concentration $> 10 \text{ mg/l}$, by specifying stratum-specific baseline hazard functions.

2.2. Analytical validation studies for the GlycA test

For the analytical validation studies, serum samples were collected from healthy volunteers in the United States of America (USA). These studies were cleared by an Institutional Review Board. For both the analytical validation and PREVENT studies, all donors signed consent forms and the studies were conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.2.1. Specimen collection and preparation

Control serum pools and specimens were purchased and prepared as previously described [14,15]. All studies were performed in NMR LipoProfile® (LipoScience) serum separator tubes (#456,293/455,232; Greiner Bio-One®), also known as LipoTubes® (LipoScience), unless otherwise indicated.

2.2.2. Acquisition of NMR spectra and data processing

NMR spectra were acquired at the Clinical Laboratory Improvement Amendments (CLIA) approved laboratory at Laboratory Corporation of America® Holdings as previously described using the Vantera Clinical Analyzer, a 400 MHz NMR spectrometer [14,15]. Typically, 2 levels of serum controls were included at the beginning and end of each specimen run. Data acquisition on the Vantera was accomplished in a similar fashion to the NMR Profiler platform with the exception that water was suppressed using the WET solvent suppression technique [14,20,21]. NMR spectral data were acquired as 3 blocks of 4 scans for a total acquisition time of 48 s and a sweep width of 4496.4 Hz and 9024 data points.

The signal peak appearing at 2.00 ppm, named GlycA, which lies outside of the spectral region used to calculate the lipoprotein information, was quantified as previously described in detail [1]. GlycA is not a

homogeneous signal from a single molecular species, but rather a composite signal arising from the superposition of slightly offset N-acetyl methyl group resonances from a subset of mobile GlcNAc residues on the glycan branches of abundant glycoproteins [6]. It is only the GlcNAc residues in $\beta(1 \rightarrow 2)$ or $\beta(1 \rightarrow 6)$ linkage with a preceding mannose residue that give rise to N-acetyl methyl resonances at the $2.00 \pm 0.01 \text{ ppm}$ GlycA position [1]. The units for the GlycA signal represent the number of GlcNAc residues in $\mu\text{mol/l}$. These particular residues can be found on many glycosylated proteins however, the glycoproteins that circulate at $> 10 \mu\text{M}$ and are highly glycosylated make the largest contributions to the composite GlycA signal (e.g. $\alpha 1$ -acid glycoprotein (orosomucoid), $\alpha 1$ -antitrypsin, $\alpha 1$ -antichymotrypsin, haptoglobin and transferrin) [1,6].

2.2.3. Assay performance testing

Sensitivity, imprecision and linearity were determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines as previously described [14,15,22–24]. For linearity testing, the high GlycA serum pool was obtained by the addition of $\alpha 1$ -acid glycoprotein, the most abundant protein that contributes to the GlycA signal [1].

2.2.4. Method and tube comparisons

Method comparison studies consistent with CLSI guidelines were performed to ensure that the performance of the GlycA test on the Vantera Clinical Analyzer was similar to the GlycA test run on the NMR Profiler [1]. Samples ($n = 631$) were tested in singlicate on the reference (Profiler) and comparator (Vantera Clinical Analyzer) NMR systems over a period of 5 days. The correlation between results generated on the two NMR platforms was evaluated using Deming regression analysis. Blood from 46 donors was drawn into three different tubes: LipoTube (serum), BD Vacutainer® serum tube (red top, no gel barrier) and K₂EDTA plasma tube. GlycA was measured for a total of 50 specimens. Results for conventional serum and EDTA plasma tubes were compared to results for the LipoTube by linear regression.

2.2.5. Reference interval and interfering substance studies

To determine the reference range for the GlycA assay, samples from healthy adult men and non-pregnant women between the ages of 18 and 84 ($n = 450$) were collected in BD Vacutainer serum tubes (red top, no gel barrier). A description of this study population has been reported [14]. The GlycA reference range was estimated using non-parametric analyses with reference limits at the 2.5th and 97.5th percentiles according to the nonparametric method described in CLSI guidelines [25]. The reference intervals for men and women were compared by assessing their median values by Mann–Whitney test. A total of 7 endogenous and 23 exogenous substances were tested in vitro for possible assay interference consistent with CLSI guidelines [26], as previously described [14,15].

2.2.6. Stability testing

2.2.6.1. Refrigerated stability. Serum samples obtained from 3 separate studies of 10 donors each ($n = 30$) were used to assess stability of GlycA. Samples were stored at 4°C and aliquots were tested daily for 12 days. Daily mean results for all donors were evaluated with acceptable differences falling within $\pm 10\%$ of the day 0 (draw day) mean.

2.2.6.2. Room temperature stability. Serum samples obtained from 12 donors were used to assess the stability of GlycA at room temperature over time. Samples were allowed to sit at room temperature for 24 h. Aliquots of the serum samples were tested at the following time points: 0, 1, 2, 4, 6, 8 and 24 h.

2.2.6.3. Freeze–thaw stability. A set of 3 pooled serum samples (low, mid and high analyte concentrations) was used to assess the stability of GlycA after 3 freeze–thaw cycles. One aliquot from each of the 3 samples

within the set was tested fresh and 3 aliquots from each were frozen at -80°C . After 24 h, all aliquots were thawed unassisted at room temperature for 1 h. Once thawed, 1 aliquot from each of the 3 samples was tested while the remaining sets of aliquots were returned to the freezer. This process continued until data for 3 freeze–thaw cycles were collected.

2.3. Statistical analysis

Analytical validation data was calculated using either Excel Analyse-it® or GraphPad Prism ver 6.0 software.

3. Results

3.1. Analytical performance of the GlycA assay

The analytical performance of the GlycA assay, as measured on the Vantera Clinical Analyzer, was assessed for the ability to accurately quantify the NMR signal at 2.00 ppm in spectra acquired from serum samples. Because the GlycA signal arises from circulating glycosylated proteins and it is not possible to remove all proteins from a serum specimen, limits of blank (LOB) and detection (LOD) could not be determined by traditional means. However, testing of five serum pools, with GlycA ranging from 57 to 166 $\mu\text{mol/l}$, gave CVs $<7.6\%$ and a functional sensitivity or limit of quantitation (LOQ) of 18 $\mu\text{mol/l}$. Because the calculated LOQ was lower than the lowest concentration measured, 57 $\mu\text{mol/l}$ should be considered the LOQ for GlycA. Serum pool samples with three varying concentrations of GlycA (low, intermediate and high) were tested for intra-assay (within-run) and inter-assay (within-lab) precision. The CV for the GlycA assay ranged from 1.3–1.8% for within-run and 1.9–2.3% for within-lab imprecision (Table 1). In order to test the ability of the assay to measure the GlycA NMR signal, the measured values ($n = 10$ serum pools) were plotted against the actual or expected concentrations. Linearity of GlycA was demonstrated throughout the reportable range of 150 to 1588 $\mu\text{mol/l}$ with a correlation coefficient (R^2) of 0.998 (Fig. 2A).

The linear regression for the GlycA data ($n = 631$), with the Profiler results as the reference method, produced a slope (95% CI) of 1.00 (0.99 to 1.01), intercept of 4.60 (-1.15 to 10.35), and correlation coefficient (R^2) of 0.983 (data not shown). Deming regression produced a slope of 1.01 (1.00 to 1.02) and an intercept of 0.09 (-5.12 to 5.30) (Fig. 2B). Differences between the GlycA values and the Deming regression line (residuals) were plotted against the estimated concentration of GlycA. The points on the residuals plot were randomly dispersed around the horizontal axis, suggesting that the results of the two assays were linearly related with no significant bias and no apparent outliers (Fig. 2C).

Serum specimens from a population of healthy individuals ($n = 450$) were used to determine the reference interval for the GlycA test.

Table 1
Within-run and within-laboratory imprecision for GlycA measured on the Vantera Clinical Analyzer.

	GlycA ($\mu\text{mol/l}$)		
	Low	Intermediate	High
<i>Within-run^a</i>			
Mean	344.1	460.9	649.9
SD	6.3	7.7	8.7
CV	1.8%	1.7%	1.3%
<i>Within-laboratory^b</i>			
Mean	342.6	463.7	667.0
SD	7.9	10.3	12.5
CV	2.3%	2.2%	1.9%

^a Based on 1 run of 20 tests.

^b Based on CLSI guidelines tested using 3 serum pools, 2 runs per day in duplicate, for 20 days (total $n = 80$).

Table 2 shows the distribution of GlycA values in this population. The mean GlycA value was $386 \pm 60 \mu\text{mol/l}$, the median was 379 $\mu\text{mol/l}$ and the reference interval (2.5–97.5th percentile) was 288–518 $\mu\text{mol/l}$ (Table 2). In women, the mean GlycA value was $395 \pm 60 \mu\text{mol/l}$, the median was 388 $\mu\text{mol/l}$, and the reference interval was 299–522 $\mu\text{mol/l}$. In men, the mean GlycA value was $370 \pm 57 \mu\text{mol/l}$, the median was 366 $\mu\text{mol/l}$ and the reference interval was 273–487 $\mu\text{mol/l}$. There was a statistically significant difference between the means and medians for men and women (both P -values <0.0001).

Seven endogenous substances normally found circulating in blood and 22 exogenous substances, previously used to test for interference with quantification of LDL-P [14] and HDL-P [15], were evaluated for potential interference with the GlycA test. Only hemoglobin, at levels which may occur in grossly hemolyzed specimens ($\geq 20 \text{ mg/dL}$), reduced GlycA concentrations by approximately 20%.

Several types of specimen collection tubes were compared to LipoTubes for their suitability in the GlycA assay. Linear regression analyses were performed and the resulting lines had slopes of 0.94 ($R^2 = 0.99$) for plain red-top serum tubes (no gel barrier) and 0.98 ($R^2 = 0.98$) for EDTA plasma tubes. Plain red-top serum showed no significant bias based on either 95% confidence intervals around the correlation slope and intercept or estimation of bias from Bland-Altman residual plots. Measurements from EDTA plasma specimens, however, were on average 3–5% lower than from serum specimens.

The stability of GlycA as measured on the Vantera Clinical Analyzer was evaluated in 30 serum samples stored for up to 12 days at 4°C . Measurements were deemed acceptable if they were within 10% of the day 0 mean GlycA. Results demonstrated GlycA was stable out to day 12 at 4°C with changes $\leq 3.7\%$ and no trend toward higher or lower values (data not shown). Additional stability studies revealed that GlycA values were stable up to 24 h at room temperature, when specimens were frozen up to 24 months and after being frozen and thawed up to 3 times. When preparing serum specimens, centrifugation could be delayed up to 24 h, when refrigerated after clotting, without experiencing a significant change in GlycA values.

3.2. Associations of GlycA with incident T2DM in PREVENT

Of the 6894 PREVENT participants that completed the second round of screening, 4524 subjects were included in this study (Fig. 1). Subjects were excluded if they were missing data for GlycA, hsCRP or information regarding prevalent T2DM, at baseline or follow-up. Subjects were also excluded if they had T2DM at baseline. After a median (interquartile range [IQR]) follow-up period of 7.3 (5.9–7.6) y, 220 incident cases of T2DM were ascertained. Baseline clinical and laboratory characteristics of the cohort are shown in Table 3. Participants with higher levels of GlycA were more likely to be older and tended to have a higher BMI, blood pressure, glucose and hsCRP levels. They also had higher TC and TG levels and lower HDL-C, and were more likely to be on lipid lowering medications. Additionally, they were more likely to be current smokers and less likely to consume alcohol.

Cox proportional hazards regression was used to evaluate the association of GlycA and hsCRP with incident T2DM (Table 4). GlycA predicted incident T2DM in models adjusted for age, sex, and additionally for BMI, alcohol intake, smoking status, lipid lowering drugs, anti-hypertensive medication, systolic blood pressure (SBP), TC, HDL-C and TG (models 1–4). The association of GlycA with incident T2DM remained present after additional adjustment for baseline glucose (model 5). When hsCRP was added to the model, the relationship of GlycA with T2DM was still significant (model 6). Comparable results were obtained when GlycA was examined per 1 SD change, although statistical significance was lost after adjustment for hsCRP (data not shown; model 6, HR 1.14 [95% CI: 0.95–1.36, $P = 0.16$]). hsCRP was also associated with T2DM in models adjusted for age, sex and additionally for BMI, alcohol intake, smoking status, lipid lowering drugs, anti-hypertensive medication and SBP (Table 4, models 1–3), but significance was lost after further

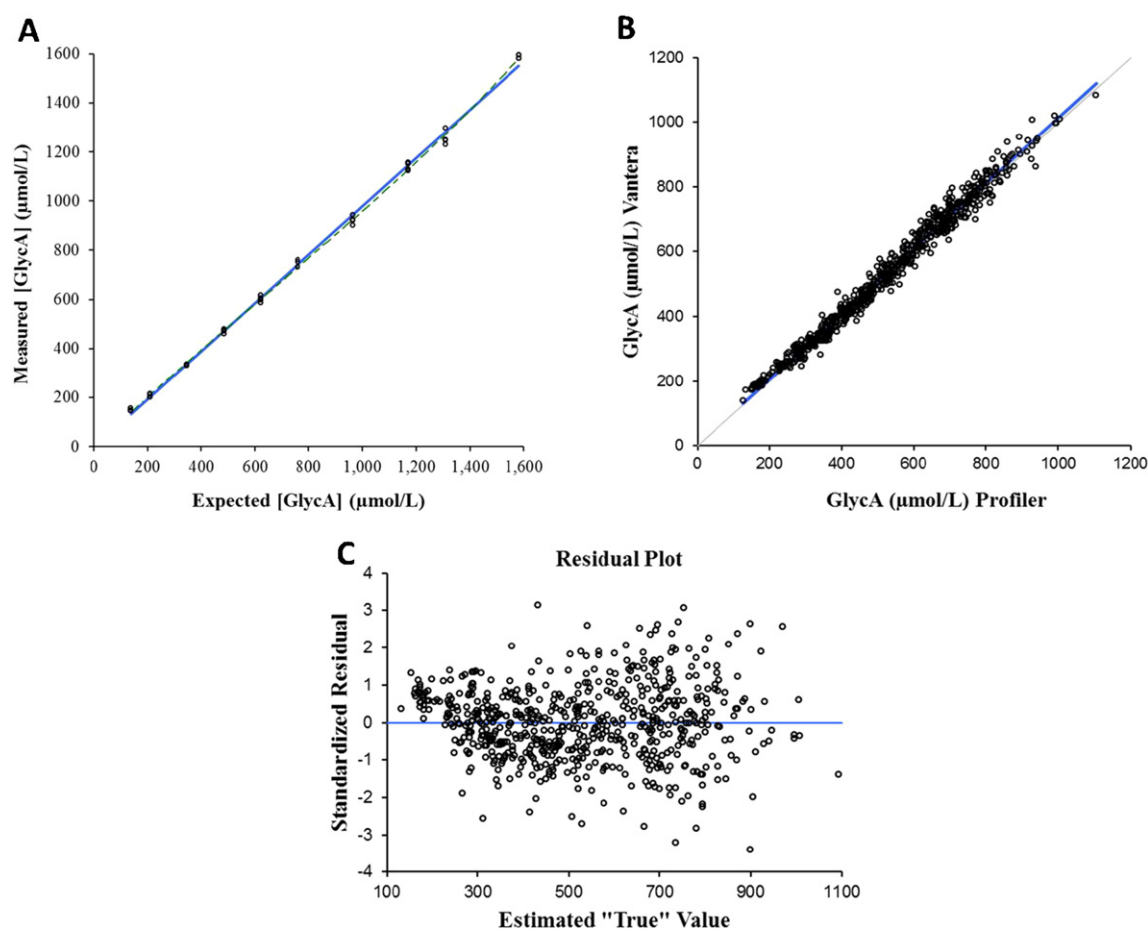


Fig. 2. A. Results of linearity testing for the Vantera GlycA assay, solid gray line = linear fit, dotted gray line = polynomial fit; B. Comparison of Vantera and NMR Profiler methods for quantification of GlycA, solid gray line = Deming fit, gray line = identity; C. Residual plot for the Deming regression.

adjustment for lipids, baseline glucose and GlycA (Table 4, models 4–6). When examined per 1 SD change, hsCRP was also not significantly associated with incident T2DM in analysis in which we accounted for GlycA (data not shown; model 6, HR 1.03 [95% CI: 0.87–1.23, $P = 0.71$]).

Furthermore, in secondary analyses in which the design of the PREVENT study with preferential inclusion of subjects with elevated urinary albumin excretion was taken into account, GlycA was still associated with incident T2DM after adjustment for hsCRP (Supplementary Table 1, model 6; HR 1.80 [95% CI: 1.05–3.07, $P = 0.03$]), whereas hsCRP did not predict T2DM after adjustment for GlycA (Supplementary Table 1, model 6; HR 1.22 [95% CI: 0.68–2.20, $P = 0.86$]).

Subsequently, we performed a joint analysis based on dichotomized subgroups with high and low GlycA or hsCRP (Table 5, Fig. 3). In crude analysis, the hazard of incident T2DM was highest in the subgroup with high GlycA and high hsCRP and lowest in the subgroup with low

GlycA and low hsCRP. After adjustment for age and sex, the hazard for incident T2DM remained the same in the subgroups with high GlycA and high hsCRP and high GlycA and low hsCRP, and was only slightly attenuated in the subgroup with high hsCRP and low GlycA compared to the subgroup with low GlycA and low hsCRP. Notably, after adjustment for age, sex, BMI, alcohol intake, smoking status, lipid lowering drugs, antihypertensive medications, SBP, lipids and baseline glucose, the hazard of incident T2DM was only significantly increased in the subgroup with high GlycA and low hsCRP.

4. Discussion

Our prospective study is the first to show that GlycA, a marker of systemic inflammation, is associated with incident T2DM in a general population of both men and women. In PREVENT participants GlycA was an independent predictor of T2DM even after adjusting for traditional diabetes risk factors and hsCRP. Recently Akinkuolie et al. reported that GlycA was associated with risk of incident T2DM in a population of initially healthy women enrolled in the WHS [11]. In the WHS study, the associations of GlycA and hsCRP with incident T2DM were attenuated but remained statistically significant after adjusting for diabetes risk factors. hsCRP remained significantly associated with incident T2DM, while the association with GlycA was no longer significant after further mutual adjustment [11]. In our study, after adjusting for diabetes risk factors as well as mutual adjustment, GlycA remained significantly associated with incident T2DM whereas the association of hsCRP was no longer significant. Moreover, in PREVENT the associations of GlycA with future T2DM were similar for men and women, while the hsCRP

Table 2
Distribution of GlycA observed in a reference population.

Percentile	GlycA (μmol/l)		
	All (n = 450)	Men (n = 158)	Women (n = 292)
0	245	245	269
2.5	288	273	299
25	346	340	350
50	379	366	388
75	420	394	434
97.5	518	487	522
100	605	605	599

Table 3

Baseline characteristics according to sex-stratified quartiles of GlycA in 4524 participants of the PREVENT study.

	Quartiles of GlycA, $\mu\text{mol/l}$				P-value
	1 Men < 301 Women < 312	2 Men 301–333 Women 312–348	3 Men 334–375 Women 349–390	4 Men \geq 376 Women \geq 391	
Participants, n	1134	1117	1146	1127	
GlycA, $\mu\text{mol/l}$	284 [267–296]	324 [315–332]	362 [351–381]	418 [398–448]	<0.001
Female, n (%)	599 (52.8)	578 (51.7)	597 (52.1)	588 (52.2)	NS
Age, years	48.7 \pm 10.5	52.0 \pm 11.6	54.2 \pm 11.8	55.4 \pm 11.7	<0.001
BMI, kg/m^2	24.5 \pm 3.4	26.0 \pm 3.7	27.0 \pm 3.9	28.0 \pm 4.7	<0.001
Smoking, n (%)					<0.001
Never	456 (40.7)	357 (32.4)	281 (24.8)	263 (23.5)	
Former	475 (42.4)	500 (45.4)	501 (44.2)	445 (39.8)	
Current	189 (16.9)	245 (22.2)	351 (31.0)	409 (36.6)	
Alcohol intake, n (%)					<0.001
Almost never	202 (18.0)	223 (20.2)	296 (26.0)	323 (28.9)	
1–4 drinks per month	192 (17.1)	195 (17.6)	196 (17.2)	188 (16.8)	
2–7 drinks per week	425 (37.8)	370 (33.5)	337 (29.6)	318 (28.5)	
\geq 1 drinks per day	305 (27.1)	318 (28.8)	309 (27.2)	287 (25.7)	
Lipid lowering drug use, n (%)	44 (3.9)	74 (6.6)	117 (10.2)	154 (13.7)	<0.001
SBP, mm Hg	118.6 \pm 15.3	123.2 \pm 17.2	127.0 \pm 19.5	129.7 \pm 18.9	<0.001
DBP, mm Hg	70.3 \pm 8.7	72.2 \pm 8.6	73.8 \pm 9.0	74.4 \pm 8.8	<0.001
Blood pressure-lowering drug use, n (%)	105 (9.3)	176 (15.8)	260 (22.7)	316 (28.0)	<0.001
hsCRP, mg/l	0.50 [0.25–0.93]	0.93 [0.53–1.55]	1.66 [0.93–3.11]	3.43 [1.80–6.85]	<0.001
Glucose, mmol/l	4.7 \pm 0.6	4.8 \pm 0.6	4.9 \pm 0.6	5.0 \pm 0.7	<0.001
TC, mmol/l	5.2 \pm 1.0	5.4 \pm 1.0	5.5 \pm 1.0	5.7 \pm 1.08	<0.001
HDL-C, mmol/l	1.3 \pm 0.3	1.3 \pm 0.3	1.2 \pm 0.3	1.2 \pm 0.3	<0.001
TG, mmol/l	0.85 [0.64–1.18]	1.02 [0.77–1.40]	1.20 [0.88–1.64]	1.35 [0.98–1.83]	<0.001

Data are expressed as mean \pm SD, median [IQR] or proportion n (%). P values are calculated by linear regression or χ^2 analysis. Diabetes was defined as fasting plasma glucose level $>$ 7.0 mmol/l or non-fasting plasma glucose level $>$ 11.1 mmol/l or use of antidiabetic medication.

Abbreviations: PREVENT, Prevention of RENal and Vascular ENd-stage Disease.

associations appeared to be somewhat stronger in women than in men. The association of hsCRP with incident T2DM in men was attenuated after adjustment for medications (model 2; HR 1.13 [95% CI: 0.94–1.35, $P = \text{NS}$]) but remained significant in women even after further adjustment for medications and lipids (model 3; HR 1.28 [95% CI:

1.04–1.59, $P = 0.02$) and was only attenuated after further adjustment for baseline glucose (model 4; HR 1.21 [95% CI: 0.97–1.51, $P = \text{NS}$]). The fact that the association of hsCRP, but not GlycA, with incident T2DM is stronger in women than in men may explain, at least in part, the differences in results between these two studies.

Table 4Association of GlycA and hsCRP with incident type 2 diabetes mellitus in the PREVENT study ($n = 4524$; 220 events).

	Quartile 1	Quartile 2	P value	Quartile 3	P value	Quartile 4	P value	P for trend*
GlycA	<306	306–340		341–383		\geq 384		
Participants (n)	1104	1147		1133		1140		
No. of 'cases' (%)	26 (2.4)	40 (3.5)		63 (5.6)		91 (8.0)		
Person years	7505	7554		7257		6998		
Crude	1	1.53 [0.93–2.51]	NS	2.52 [1.60–3.99]	<0.001	3.81 [2.46–5.89]	<0.001	<0.001
Model 1	1	1.40 [0.86–2.30]	NS	2.24 [1.41–3.55]	0.001	3.39 [2.18–5.28]	<0.001	<0.001
Model 2	1	1.22 [0.74–2.00]	NS	1.75 [1.09–2.79]	0.02	2.14 [1.34–3.41]	0.001	<0.001
Model 3	1	1.17 [0.71–1.92]	NS	1.52 [0.95–2.44]	NS	1.82 [1.13–2.91]	0.01	0.004
Model 4	1	1.08 [0.66–1.77]	NS	1.33 [0.82–2.13]	NS	1.55 [0.97–2.48]	0.07	0.005
Model 5	1	1.23 [0.73–2.06]	NS	1.64 [1.01–2.66]	0.05	1.77 [1.10–2.86]	0.02	0.01
Model 6	1	1.22 [0.72–2.05]	NS	1.61 [0.98–2.66]	NS	1.71 [1.00–2.92]	0.05	0.04
hsCRP	<0.58	0.58–1.25		1.26–2.82		\geq 2.83		
Participants (n)	1132	1130		1134		1128		
No. of 'cases' (%)	20 (1.8)	41 (3.6)		74 (6.5)		85 (7.5)		
Person years	7703	7485		7148		6977		
Crude	1	2.11 [1.24–3.60]	0.006	4.03 [2.46–6.60]	<0.001	4.75 [2.92–7.74]	<0.001	<0.001
Model 1	1	1.85 [1.08–3.16]	0.03	3.27 [1.98–5.39]	<0.001	3.93 [2.40–6.45]	<0.001	<0.001
Model 2	1	1.43 [0.83–2.45]	NS	2.12 [1.27–3.53]	0.004	2.10 [1.25–3.54]	0.005	0.02
Model 3	1	1.37 [0.80–2.36]	NS	1.94 [1.16–3.24]	0.01	1.88 [1.11–3.17]	0.02	NS
Model 4	1	1.18 [0.68–2.03]	NS	1.64 [0.98–2.75]	NS	1.56 [0.92–2.64]	NS	NS
Model 5	1	1.14 [0.65–1.99]	NS	1.58 [0.93–2.68]	NS	1.47 [0.86–2.51]	NS	NS
Model 6	1	1.08 [0.62–1.89]	NS	1.45 [0.84–2.48]	NS	1.20 [0.67–2.17]	NS	NS

Hazard ratios were derived from Cox proportional hazards regression models. TG and hsCRP were log transformed when used as a continuous variable in the analysis.

Model 1: crude model + age, sex.

Model 2: model 1 + BMI, alcohol intake, smoking status.

Model 3: model 2 + lipid lowering drugs, anti-hypertensive medication and SBP.

Model 4: model 3 + TC, HDL-C, TG.

Model 5: model 4 + baseline glucose.

Model 6: model 5 + hsCRP (for GlycA analysis) and GlycA (for hsCRP analysis).

Abbreviations: PREVENT, Prevention of RENal and Vascular ENd-stage Disease.

* Tests of trend across increasing quartiles were conducted by assigning the median for each quartile as its value and treating this as a continuous variable.

Table 5

Joint analysis of GlycA and hsCRP categories on incident type 2 diabetes mellitus.

	Low GlycA Low hsCRP	Low GlycA High hsCRP	P value	High GlycA Low hsCRP	P value	High GlycA High hsCRP	P value
Participants (n)	2938	468		458		660	
No. of 'cases' (%)	101 (3.4)	29 (6.2)		34 (7.4)		56 (8.5)	
Crude	1	1.89 [1.25–2.86]	0.002	2.32 [1.57–3.42]	<0.001	2.74 [1.98–3.80]	<0.001
Model 1	1	1.68 [1.11–2.54]	0.02	2.16 [1.46–3.19]	<0.001	2.54 [1.82–3.53]	<0.001
Model 2	1	1.19 [0.77–1.83]	NS	1.57 [1.04–2.36]	0.03	1.24 [0.86–1.78]	NS

Higher levels were defined as greater than the upper quartile for GlycA >384 $\mu\text{mol/l}$ and hsCRP >2.83 mg/l.

Model 1: crude model + age, sex.

Model 2: model 1 + BMI, alcohol intake, smoking status, lipid lowering drugs, anti-hypertensive medication, SBP, baseline glucose and TC, HDL-C, TG.

GlycA may provide complementary and possibly superior information to hsCRP for the prediction of future T2DM. GlycA is unique in that it is an NMR signal derived from residues within the carbohydrate side-chains of multiple acute phase reactants [1]. Not only are the synthesis and secretion of these proteins increased during inflammation, but their glycan structures are dynamically altered [27–30]. For example, the carbohydrate side-chains of α 1-acid glycoprotein, one of the acute phase glycoproteins captured in the GlycA signal, become highly branched and contain additional GlcNAc residues in the context of chronic inflammation and cancer [28,31]. It is plausible that GlycA represents a composite biomarker with the potential to have disease associations that differ, or are even stronger, than individual inflammatory markers [2]. In this regard, it is also relevant that GlycA is less variable within subjects than hsCRP [1]; in our study, GlycA remained significantly associated with incident T2DM even after adjusting for diabetes risk factors and hsCRP.

Low grade inflammation is known to trigger the development of insulin resistance and loss of β -cell function, both of which are proposed to be implicated in the pathogenesis of T2DM (for review: [32–35]). Accumulating evidence suggests that carbohydrate metabolism and the immune system are intricately linked. Nutritional excess leads to enhanced systemic inflammation via multiple mechanisms including obesity and ensuing adipose tissue inflammation and alterations in the intestinal microbiome [32]. In turn, tissue and circulating inflammatory mediators may be causally implicated in the development of insulin resistance and β -cell dysfunction, factors that are key for the progression to T2DM [32–35]. Previous studies revealed that there are increases in individual components of the innate immune system, including cytokines, complement factors and acute phase proteins, in subjects with T2DM [36]. In addition, several of these inflammatory markers have been shown to be associated with incident T2DM [37–41]. Therefore, it makes biological sense that GlycA, a unique multi-marker of systemic inflammation, would be independently related to diabetes risk.

It may be possible to combine GlycA with other NMR-measured metabolic and lipoprotein biomarkers of diabetes risk in order to increase the specificity of the prediction for progression to T2DM. For example, specific alterations in the lipoprotein particle distribution occur with insulin resistance and metabolic syndrome [42–44]. In addition, branched chain amino acids such as valine are associated with incident T2DM [45–47]. Because GlycA, valine and lipoprotein particle information can be quantified simultaneously from the same NMR spectra of serum or plasma, it would be straightforward to combine these pieces of information in a single algorithm that would predict a patient's risk of progressing to T2DM. Studies investigating the utility of GlycA in this regard are ongoing.

The successful development of a high-throughput method to measure GlycA on a fully automated platform allows NMR technology dissemination into the routine clinical laboratory setting and creates the opportunity for NMR-based testing across a broader range of clinical applications [14,15]. The performance characteristics of the GlycA test on the presently used device underscores that it is suitable for clinical applications. Furthermore, high-throughput measurements of GlycA allow both physician use as well as efficient exploration of additional disease associations in large clinical studies like PREVENT.

We acknowledge several strengths and weaknesses in our study. Our study included a large number of participants. Notably, the PREVENT study was designed to study the impact albuminuria on renal and cardiovascular outcome. Thus, subjects were preferentially recruited on the basis of their urinary albumin concentrations; approximately two thirds having urinary albumin concentrations ≥ 10 mg/l and one third having <10 mg/l at the start of this cohort study. For this reason we performed a secondary analysis taking account of the design of the PREVENT study. This analysis revealed no differences in the results or conclusions that were drawn for the entire population. This finding is also relevant because albuminuria itself may confer increased risk of diabetes development [48]. In addition, it should be appreciated that most PREVENT participants were of Caucasian descent,

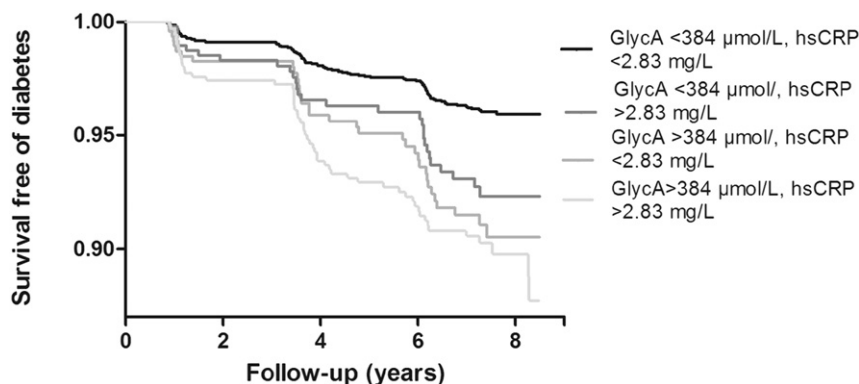


Fig. 3. Kaplan–Meier curves of diabetes according to joint levels of GlycA and hsCRP. High levels of GlycA were defined as higher than top quartile, i.e. >384 $\mu\text{mol/l}$ for GlycA and >2.84 mg/l for hsCRP.

possibly limiting extrapolation of our findings to other populations. Nonetheless, we were able to extend the results obtained in an initially healthy large population of US women [11].

In conclusion, GlycA, an assay that is available for testing on the Vantera® Clinical Analyzer, may be useful as a diagnostic indicator for risk of T2DM, alone or in combination with other NMR-measured analytes, particularly in a population with abnormal urinary albumin.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.cca.2015.11.001>.

Author's conflict of interest disclosure

MAC, JWD, SPM, IS and JDO are employees of LipoScience, Laboratory Corporation of America® Holdings, a company that is marketing the Vantera® Clinical Analyzer and the GlycA assay for clinical diagnostic use.

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